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Blood and Charcoal Added to Acidified Agar Media Promote the Growth of *Mycobacterium genavense*

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Ten different agar media were tested for the in vitro growth of Mycobacterium genavense *in primary cultures and in subcultures from BACTEC vials. These agar media were based on Middlebrook 7H9, 7H10 and 7H11, and supplemented with additives: mycobactin J, yeast extract, charcoal, or defibrinated sheep blood. Some media were acidified with phosphoric acid to a final pH of 6.2 ± 0.2. Fourteen* M. genavense *strains from nude mouse organs as well as one decontaminated clinical specimen (from a bird) were tested. The optimal medium for primary cultures of* M. genavense *was Middlebrook 7H11 acidified to pH 6.2 ± 0.2 and supplemented with charcoal and sheep blood: on this medium, all strains produced colonies within 6–12 weeks of incubation in numbers approaching the number of bacilli inoculated. It was also the only medium to support the growth of the decontaminated clinical specimen. Added blood and charcoal appeared not as essential for subcultures as for primary cultures. Three media supported the growth of all strains within 1 month incubation: they were acidified, and were supplemented with yeast extract or pancreatic digest of casein, and with either blood or charcoal.*

INTRODUCTION

Mycobacterium genavense was first described by Hirschel et al. (1990). The organism did not grow on solid media, a very low growth index (GI) was measured in radiometric Middlebrook 7H13 (BACTEC System), whereas subcultures remained negative. Better growth was reported in acidified liquid media such as BACTEC vials at pH 6.0 (Böttger et al. 1993; Hoop et al. 1993, 1996; Realini et al. 1997; Siddiqi et al. 1993; Tortoli et al. 1994). Primary cultures on solid media such as Löwenstein-Jensen (LJ) and Middlebrook 7H10 or 7H11 are not recommended for the isolation of *M. genavense* (Böttger et al. 1993; Hoop et al. 1996). In 1992, Jackson et al. reported the development of colonies of *M. genavense* on Middlebrook 7H9 with agar, charcoal and yeast extract added, whereas Coyle et al. (1992), recommended Middlebrook 7H11 supplemented with Mycobactin J. Subcultures from positive BACTEC vials were obtained by Jackson et al. (1992), after 6 weeks to 6 months, and by Coyle et al. (1992), after 3 to 9 weeks. In the description of *M. genavense* sp nov (Böttger et al. 1993; Coyle et al. 1992), the growth of *M. genavense* on solid media was reported as poor, extremely slow and variable. More recently, Maier et al. (1995) reported more luxuriant growth of *M. genavense* on Middlebrook 7H10 supplemented with human blood compared with Middlebrook 7H11 supplemented with Mycobactin J.

Polymerase chain reaction and the BACTEC System are the optimal detection methods for *M. genavense*, but not all laboratories, particularly not veterinary and zoological laboratories, have access to these systems. It is therefore desirable to optimize the

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isolation of *M. genavense* and to characterize the species phenotypically. We here report results of studies on the growth of *M. genavense* on 10 different agar media.

MATERIALS AND METHODS

Mouse inoculation

Eight-week-old female congenitally athymic BALB/c mice (IFFA Credo, Lyon, France) were inoculated intraperitoneally with 0.25 mL suspensions of *M. genavense* containing at least 10^5 acid-fast bacilli (AFB) per mL (maximum 10^6) (Hirschel et al. 1990). The mice were killed 8 to 9 months after inoculation, and the spleens and lungs kept at −70°C.

Fourteen *M. genavense* strains obtained from nine AIDS patients (six from Switzerland [strains: Institute of Tropical Medicine (ITM) 95-608, ITM 95-975, ITM 96-1283, ITM 96-1438, ITM 96-1439, ITM 96-1799], three from USA [ITM 96-823, ITM 97-75, ITM 97-76]) and from five zoo birds from Antwerp, Belgium (ITM 95-614, ITM 95-615, ITM 95-781, ITM 96-114, ITM 96-560) were used.

Preparation of the mouse tissue suspensions

The organs were thawed, weighed, minced with sterile scissors, and homogenized with a pestle and mortar in 20% (wt/vol) phosphate-buffered saline (PBS). The number of AFB were counted by the method of Shepard and Mc Rae (1968). Dilutions (10^7 to 10^2 AFB/mL) of the tissue homogenate were made in PBS. No decontamination was performed.

Clinical specimen

The liver (kept at −20°C) of an African silver-bill *(Euodice cantans)* kept in the Antwerp Zoo was sent to us because it did not grow on Ogawa and Löwenstein-Jensen media, although the inoculum was very rich in AFB. These AFB were identified as *M. genavense* by characterization of specific sequences in the *rrn* operon (data not shown). The specimen was thawed, minced and homogenized with a pestle and mortar in PBS. The tissue homogenate was centrifuged differentially to remove the larger tissue debris (100 × g, 5 min., 10°C). The supernatant was then centrifuged at 2'700 × g, 45 min., 10°C, and the resulting pellet containing the AFB resuspended in PBS. AFB were counted as described above. The bacterial suspension was divided into 4 aliquots, each decontaminated by one of the following procedures: (i) sodium dodecyl sulfate (SDS) (Nolte and Metchock, 1995), (ii) N-acetyl-L-cysteine-sodium hydroxide (NALC) (Nolte and Metchock 1995), (iii) Sodium hydroxide 4% (Petroff 1915), and (iiii) hydrochloric acid (HCl) 1N followed by neutralization with sodium hydroxide 4%.

Solid media

Formulation of the culture media and their pH are presented in Table 1. The Middlebrook media (7H9, 7H10, 7H11) were prepared according to the manufacturer's instructions (Difco Laboratories) with respect of glycerol (0.2% v/v for 7H9; 0.5% v/v for 7H10 and 7H11) and Enrichments (ADC for 7H9; OADC for 7H10 and 7H11). Middlebrook 7H11 contains 1 g/lt of pancreatic digest of casein absent from Middlebrook 7H9 and 7H10. Some media were supplemented with different additives or/and acidified to a final pH of 6.2 ± 0.2 (see Table 1). The following additives were used: yeast extract (YE) (Difco Laboratories) 1% (w/v), mycobactin J (MJ) (Rhône Mérieux) 2 μg/mL, charcoal (C) (Sigma) 0.2% (w/v), and defibrinated sheep blood (SB) (Ego Laboratories)

TABLE 1 Composition and pH of the Middlebrook Agar Media

	Additives				Acidification	pH (±0,1) (55°C)		
Abbreviation[a]	Charcoal	Sheep blood	Yeast extract	Mycobactin J	Phosphoric Acid	Before autoclave	Final	Reference
7H11	−	−	−	−	−	ND	6,7	
7H11 MJ	−	−	−	+	−	ND	6,7	Coyle et al. 1992
7H11 MJ AP	−	−	−	+	+	6,1	6,2	
7H11 SB	−	+	−	−	−	ND	6,8	
7H11 SB AP	−	+	−	−	+	5,9	6,3	
7H11 SB C	+	+	−	−	−	ND	6,8	
7H11 SB C AP	+	+	−	−	+	5,9	6,3	
7H10 SB AP	−	+	−	−	+	5,9	6,3	
7H9 CYE	+	−	+	−	−	ND	6,6	Jackson et al. 1992
7H9 CYE AP	+	−	+	−	+	6,1	6,2	

a: Middlebrook media (7H9, 7H10 or 7H11) are supplemented (see Material and Methods) with the following components: AP, acidification with phosphoric acid; C, charcoal; MJ, Mycobactin J; SB, sheep blood; YE, yeast extract
ND, not done

10% (v/v). Middlebrook 7H9 was solidified with 1.5% (w/v) agar (Difco Laboratories). The MJ and the SB were added after sterilisation and cooling of the medium at 55°C. The "acid" media were prepared as follows: the agar media were acidified with phosphoric acid 85% (AP) (Merck) at 55°C before sterilization to a pH such that after addition of the additives the final pH was as described in Table 1. Slants (5 mL medium/tube) were prepared in tubes with silicone caps.

Primary cultures

A total of 0.2 ml of undecontaminated suspensions prepared from mouse organs containing 10^2 to 10^7 AFB/mL and of the decontaminated clinical specimen were inoculated onto the agar slants. They were closed tightly, incubated horizontally overnight and then vertically, at 37°C in air for 3 months. The suspensions (0.1 mL) were also inoculated in a Middlebrook 7H12 pH 5.9 ± 0.2 without additives (Realini et al. 1997) and read in a BACTEC 460 TB instrument as recommended by the Manufacturer.

Subcultures

Positive BACTEC vials (GI 999) were subcultured on the different solid media and incubated as described above.

RESULTS

Figure 1 shows the number of strains developing on the different agar media as a function of the inocula. The optimal medium was Middlebrook 7H11 acidified with phosphoric acid, supplemented with sheep blood and charcoal (7H11 SBCAP): all strains grew on it within 6–12 weeks depending on the inoculum, and produced a number of colonies approaching the number of AFB inoculated. The acidified media described by Coyle et al. (1992) and Jackson et al. (1992) were not superior to the neutral media described by these authors, although the acidification allowed earlier detection. Mycobactin J added to Middlebrook 7H11 (Coyle et al. 1992) allowed earlier growth especially for high inocula of 10^6 and 10^5 AFB/mL. Addition of blood to Middlebrook 7H11 (7H11SB) without previous modification of the pH was the least efficient with growth of only one strain after inoculation of 10^7 AFB/mL. The addition of charcoal (7H11 SBC) improved it impressively: positive results being obtained with seven strains after an inoculation of 10^7 AFB/mL, and with all bacillary concentrations for one strain. Acidified blood containing media produced relatively good results both in terms

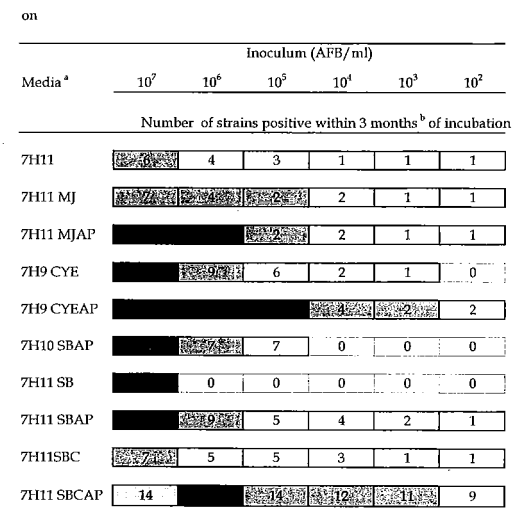

FIGURE 1 Primary cultures of 14 *M. genavense* (from undecontaminated nude mouse organs) on the different agar media. a: Middlebrook media (7H9, 7H10 or 7H11) are supplemented (see Materials and Methods) with the following components: AP, acidification with phosphoric acid; C, charcoal; MJ, Mycobactin J; SB, sheep blood; YE, yeast extract. b: growth in < 6 weeks ▨, growth after 6 to 8 weeks incubation ▩, growth after 8 weeks and ≤ 10 weeks incubation □, growth after 10 weeks and ≤ 12 weeks incubation □, no growth within 3 months .

of the number of strains developing and time of detection, Middlebrook 7H11 being superior to Middlebrook 7H10 as seen by comparing the results obtained with 7H11 SBAP and 7H10 SBAP.

Primary cultures of the clinical specimen (9.6 10^6 AFB/mL) decontaminated with the 4 different decontamination procedures were positive on the 7H11 SBCAP medium only. Growth was detected after 2 months incubation and colonies developed luxuriantly during the next 2 weeks. The number of the colonies developing differed with the decontamination procedure applied: confluent growth was obtained after decontamination with either NALC or HCl, 200–500 colonies after SDS and 100–200 after Petroff treatment.

Primary cultures in BACTEC vials from the 14 nude mouse organs and the clinical sample were subcultured on the different solid media when the GI reached 999 (mean days, 18; range, 13–30 days). Figure 2 shows the number of positive subcultures on the agar media as well as the time required to obtain visible colonies. Three media—7H9 CYEAP, 7H11 SBAP, and 7H11 SBCAP—were optimal giving rise to positive cultures for all strains within 4 weeks of incubation. All subcultures on 7H9 CYE, 7H11 SBC, and 7H10 SBAP were also positive, but after a somewhat longer incubation time (6, 6, and 5 weeks, respectively).

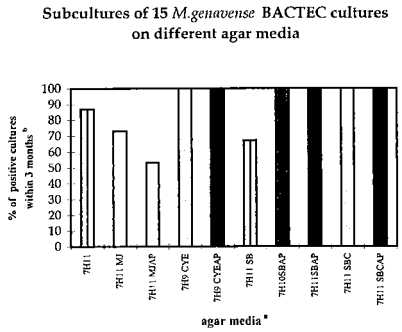

FIGURE 2 a: Middlebrook media (7H9, 7H10 or 7H11) are supplemented (see Materials and Methods) with the following components: AP, acidification with phosphoric acid; C, charcoal; MJ, Mycobactin J; SB, sheep blood; YE, yeast extract b: growth in < or = 4 weeks ■, growth after 5 weeks ▒, growth after 6 weeks □, growth after 7 weeks ▥, growth after 8 weeks □.

DISCUSSION

Numerous attempts have been made to obtain growth of *M. genavense* on solid media particularly in primary cultures. The organism failed to grow on Coletsos, Ogawa, and Stonebrink media, even after a 6-month incubation (Hirschel et al. 1990). Nadal et al. (1993) reported absence of growth on LJ, Middlebrook 7H10, Middlebrook 7H11, egg-based Gottsacker and Herrold medium. Jackson et al. (1992) reported absence of growth on LJ containing 0.4% sodium pyruvate and on egg yolk agar. Hoop et al. (1993) reported growth of *M. genavense* on Middlebrook 7H10 and Middlebrook 7H11 of one strain of *M. genavense* out of six strains studied, and no growth on LJ even after a 12-week incubation. In a later study, colonies were obtained from 2 isolates of *M. genavense* out of 34 on LJ, another isolate was obtained on Middlebrook agar (Hoop et al. 1996). Coyle et al. (1992) reported primary cultures of *M. genavense* on Middlebrook 7H11 for two specimens positive for AFB: a bone marrow containing rare AFB, positive after 60 days, and a spleen containing numerous AFB yielding pinpoint colonies after 25 days. Both specimens did not grow on LJ. Maier et al. (1995) reported primary cultures of *M. genavense* from non decontaminated human specimens (sputum, lung, liver, spleen, and kidney) on Middlebrook 7H10, supplemented with 10% human blood: pinpoint colonies were detected after a 4-week incubation at 37°C in air with or without carbon dioxide added. In our laboratory, introductory studies showed the uselessness of egg media such as LJ, Stonebrink and Ogawa (pH 6.0); a very high inoculum ($\geq 10^7$ AFB/mL) being necessary to obtain very few colonies after a 3- to 5-month incubation, even with undecontaminated specimens. The failure of the conventional solid media (LJ, Middlebrook 7H10 and 7H11) to support the growth of most strains in primary culture led us to compare different additives or modifications of the agar media.

The pH range of the media currently used in the mycobacteriology laboratories is 6.6 ± 0.2 for agar media to 7.0 for egg media, Ogawa egg yolk medium has a pH of 6.0. Acidified liquid Middlebrook 7H12, pH 5.9 ± 0.2 has been reported to improve growth of *M. genavense* by numerous authors (Böttger et al. 1993; Hoop et al. 1993, 1996; Realini et al. 1997; Siddiqi et al. 1993; Tortoli et al. 1994). The optimal pH growth range of *M. genavense* is 5.8 to 6.4 as shown by preliminary experiments (data not shown), which is somewhat wider than the pH range of optimal growth of *M. lepraemurium* (pH 5.8 to 6.1), another difficult-to-grow species (Portaels and Pattyn 1982). The pH of the "acid" media in the present experiments is 6.2 ± 0.2, compared with 6.7 ± 0.2 for the "neutral" media. Acidification of the agar media with phosphoric acid allowed earlier growth detection, as shown in Figure 1. Successful acidification of Middlebrook 7H12 with phosphoric acid was reported by Heifets and Iseman, 1985 for testing susceptibility of mycobacteria to pyrazinamide. The addition of blood to the acidified Middlebrook 7H11 was superior for the recovery of *M. genavense* as compared with Mycobactin J (see Figure 1: 7H11 MJAP versus 7H11 SBAP). Middlebrook 7H11 contains 1 g/lt of pancreatic digest of casein absent from Middlebrook 7H10 and 7H9. Better growth of *M. genavense* is observed on 7H11 SBAP versus 7H10 SBAP. Enzymatic casein hydrolysate was added to the Middlebrook media to stimulate the difficult-to-grow strains of *M. tuberculosis* (Cohn et al. 1968). Jackson et al 1992 intended to provide additional nutrients by the addition of yeast extract, and charcoal to absorb toxic substances. Charcoal is a known detoxifier: it neutralizes hydrogen peroxide and prevents the accumulation of photochemically generated toxic oxygen derivatives (Bolton et al. 1984). Charcoal may also absorb CO_2 and modify the surface tension (Feeley et al. 1979). Addition of charcoal to the media is quite beneficial for the growth of *M. genavense* (e.g., 7H11 SBC versus 7H11 SB; 7H11 SBAPC versus 7H11 SBAP), allowing earlier detection of growth and better recovery of low inocula. Blood is a commonly used additive in bacteriological media. This is not the case in the routine mycobacteriology laboratory, where media containing blood are rarely used. However blood containing media have been reported for the growth of *M. tuberculosis* (Tarshis, 1953), *M. bovis* (Cousins et al. 1989), *M. haemophilum* (Damato and Collins, 1984; McBride et al. 1992), and more recently for *M. genavense* (Maier et al. 1995). Blood may act as an enrichment factor or improve the aerotolerance of *M. genavense*. Colonies on blood containing media were dirty-brown, com-

pared to the white to buff-colored colonies on Jackson or Coyle media. We observed a positive impact of low oxygen concentrations on the metabolic activity of *M. genavense* in Middlebrook 7H12 in the BACTEC System, the organism being microaerophilic rather than aerobic (Realini et al. 1998). The addition of blood and charcoal to the media may thus enhance the oxygen tolerance of *M. genavense.* Blood contains catalase and superoxide dismutase, which can destroy hydrogen peroxide and superoxides anions appearing in the media when exposed to air and light. The toxicity of oxygen for microaerophilic bacteria can be overcome on solid or in liquid media by very large inocula. Growth failures resulted from inocula with low numbers of bacilli (Tortoli et al. 1994), whereas the rare primary cultures of *M. genavense* reported, were from specimens very rich in AFB (Coyle et al. 1992; Hoop et al. 1993, 1996; Maier et al. 1995). The medium we propose allows recovery of *M. genavense* from low inocula, and was the only one to support the growth of *M. genavense* from a decontaminated clinical specimen. Decontamination methods could also adversely affect *M. genavense* viability (Realini et al. 1998), as it was recently reported for *M. ulcerans* (Palomino and Portaels, 1998).

Liquid cultures of *M. genavense* in the BACTEC System were obtained earlier than on any solid media tested: all the strains reached GI 999 within 1 month (mean, 18 days; range, 13–30 days), and were subcultured on the solid media. Six solid media supported the growth of all strains. The three media (7H9 CYEAP, 7H11 SBAP, and 7H11 SBCAP) giving rise to 100% positivity within 1 month incubation were all acidified to pH 6.2 ± 0.2, contained nutrient additives such as yeast extract or pancreatic digest of casein, and were supplemented with either charcoal or blood or both. The addition of both blood and charcoal was not as essential for subcultures as for primary cultures. The incubation time of *M. genavense* on the medium described by Coyle et al. (1992) (7H11 MJ) was 2 months. Böttger et al. (1993) reported that after inoculation with a broth culture, visible growth of *M. genavense* on this medium required 3 to 9 weeks. In our study, the addition of Mycobactin J to Middlebrook 7H11 was not beneficial when considering the number of strains growing on this medium, but it allowed earlier detection of the colonies. Our solid media were incubated in air at 37°C. We did not have an incubator with 10% CO_2, as used by Coyle et al. (1992) and Jackson et al. (1992), but recently Maier et al. (1995) reported no difference in the growth of *M. genavense* in air or air supplemented with 10% CO_2.

The number of infections caused by *M. genavense* may be underestimated because of its poor in vitro growth (Böttger 1994). Pechère et al. (1995) reported that infection with *M. genavense* was responsible for 12.8% of disseminated nontuberculous mycobacterial infections in patients with HIV. Reliable in vitro cultures of *M. genavense* are necessary for diagnostic, physiologic, and metabolic studies. The ideal culture medium should support rapid growth of small numbers of mycobacteria, even from decontaminated specimens, and colonies should be easily detected. In the present study, Middlebrook 7H11 supplemented with blood and charcoal and acidified to pH 6.2 ± 0.2 detected more isolates than any of the other media tested. It was also superior in terms of faster detection. The modified color of the media resulting from the addition of blood and charcoal improves the visualization of the colonies. We propose that other researchers evaluate this medium with clinical specimen from both human and bird origin.

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